

UC Berkeley

UC Berkeley Previously Published Works

Title

Site-Selective Functionalization of (sp³)C–H Bonds Catalyzed by Artificial Metalloenzymes Containing an Iridium-Porphyrin Cofactor

Permalink

<https://escholarship.org/uc/item/148429rp>

Journal

Angewandte Chemie, 131(39)

ISSN

0044-8249

Authors

Gu, Yang
Natoli, Sean N
Liu, Zhennan
et al.

Publication Date

2019-09-23

DOI

10.1002/ange.201907460

Peer reviewed

Site-Selective Functionalization of (sp³)C-H Bonds Catalyzed by Artificial Metalloenzymes Containing an Iridium-Porphyrin Cofactor

Yang Gu^{†,§}, Sean N. Natoli^{†,§}, Zhennan Liu^{†,§}, Douglas S. Clark^{#Δ*}, and John F. Hartwig^{†§*}

[†]Department of Chemistry, University of California, Berkeley, California 94720, United States.

[§]Chemical Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94720, United States.

[#]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, California 94720, United States.

^ΔMolecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, California 94720, United States.

Supporting Information Placeholder

ABSTRACT: The selective functionalization of one C-H bond over others in nearly identical steric and electronic environments can facilitate the construction of complex molecules. We report site-selective functionalizations of C-H bonds, differentiated solely by remote substituents, catalyzed by artificial metalloenzymes (ArM's) that are generated from the combination of an evolvable P450 scaffold and a noble metal-porphyrin cofactor. The generated systems catalyze the insertion of carbenes into the C-H bonds of a range of phthalan derivatives containing substituents that render the two methylene positions in each phthalan inequivalent. These reactions occur with site-selectivities up to 17.8:1 and, in most cases, with pairs of enzyme mutants that preferentially form each of the two constitutional isomers. The reactions occurred in yields up to 79%, corresponding to TON up to 2286. By using iridium, we minimized the requisite number of directed evolution steps to adjust the reactivity and selectivity of the enzyme active site. This study demonstrates the potential of artificial metalloenzymes to catalyze C-H bond functionalization with site-selectivity that is difficult to achieve with small-molecule catalysts.

INTRODUCTION

C-H bond functionalization has changed the logic of chemical synthesis by enabling the direct addition of functional groups to a wide range of available starting materials.¹⁻⁴ Small-molecule, transition-metal catalysts can lead to site-

selective C-H bond functionalization,⁵⁻⁹ but many of the strategies for achieving selectivity rely on localized steric effects, directing groups, or both. Thus, it is challenging for small-molecule catalysts to distinguish between C-H bonds with similar local steric and electronic properties rendered inequivalent by the lack of symmetry of a molecule. However, enzymes, such as cytochromes P450 (CYPs), catalyze the oxidation of C-H bonds that are nearly equivalent, with high site-selectivity.^{10, 11} Such reactions often occur within the biosynthetic pathways of complex natural products, such as terpenes, alkaloids or steroids, all of which contain an abundance of similar C-H bonds.¹²

Although they can react with high selectivity, natural enzymes install a limited range of functional groups during biosynthesis. One strategy to expand the scope of reactions catalyzed by enzymes and improve the efficiency by which these reactions can occur is to conduct directed evolution. Directed evolution has proven to be a powerful methodology that allows chemists to extend the fitness of biocatalysts toward unnatural reactions.¹³⁻¹⁵ Through directed evolution, in which many rounds of iterative mutagenesis are conducted, hemoproteins have been repurposed to catalyze a range of unnatural atom transfer reactions with high activity and excellent selectivity.¹⁶⁻¹⁸ A complementary strategy to expand the scope of known abiological transformations is to construct

artificial metalloenzymes (ArMs). These systems incorporate unnatural cofactors into evolvable protein scaffolds.¹⁹⁻²² The resulting ArMs are capable of catalyzing abiological reactions, often with high selectivity. Several ArMs have been constructed by exploiting the biotin-streptavidin technology,²³⁻²⁵ unnatural amino acid anchoring,²⁶⁻²⁸ and the formal replacement of the native heme cofactor with synthetic porphyrin cofactors containing noble metals.²⁹⁻³⁴

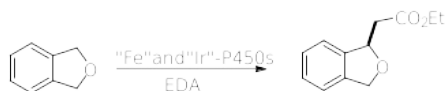
Many of these ArMs catalyze reactions that lack biological counterparts with high activity and stereoselectivity. However, few ArMs catalyze intermolecular, site-selective transformations at C-H bonds.¹⁹ Our laboratory reported the first example of carbene insertion into (sp³) C-H bonds, including intermolecular functionalizations, catalyzed by biocatalysts containing an Ir(Me)-MPIX cofactor.³⁵ Such reactivity provides a foundation to evaluate whether Ir(Me)-ArMs could be evolved to catalyze site-selective reactions of C-H bonds that are nearly equivalent and are known to yield a mixture of products with small-molecule catalysts.³⁶ Phthalan derivatives were chosen as model substrates because they have two sites that contain (sp³) C-H bonds with similar steric and electronic environments. In addition, the unsubstituted phthalan undergoes reaction with carbene precursors catalyzed by a P450 containing Ir(Me)-MPIX (Scheme 1).³⁵

Scheme 1. Intermolecular Insertion of Acceptor-only Carbenes into (sp³)C-H Bonds with Small-Molecule Catalysts and Biocatalysts.

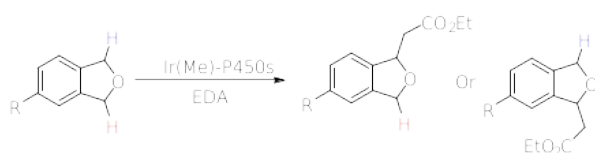
A. Small-molecule catalyst for intermolecular C-H functionalization



B. Laboratory-evolved biocatalyst for enantioselective C-H functionalization



C. This work: Regio- and chemo-selective C-H functionalization using Ir(Me)-P450



Here we report that 4-substituted phthalans react to give products that are distinguished only by the position of a remote substituent and react with pairs of Ir(Me)-CYP119 mutants to give the opposite constitutional isomer as the major

product for most of the derivatives. We also show that the reactions occur with high chemoselectivity for C-H bonds over typically-reactive functional groups, such as N-H bonds, and in select cases with high enantioselectivity.

RESULTS AND DISCUSSION

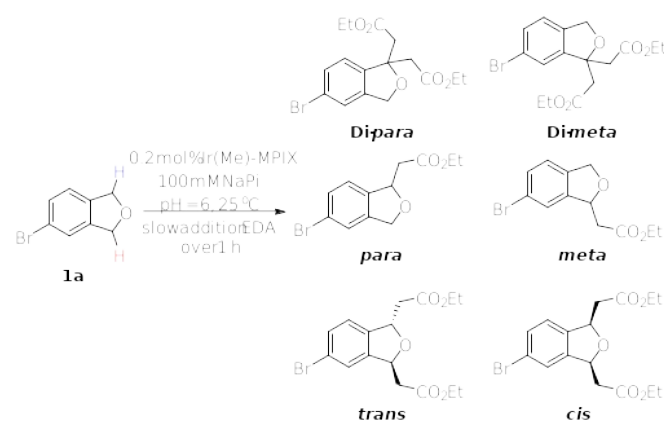
Reactivity of the free Ir(Me)-MPIX cofactor and engineered Ir(Me)-hemoproteins.

To determine the role of the protein scaffold in controlling regioselectivity, we first assessed the reactivity and site-selectivity of the free Ir(Me)-MPIX cofactor for the insertion of the carbene from ethyl 2-diazoacetate (EDA) into the C-H bond of a 4-substituted phthalan. To do so, we studied the reaction of 4-bromophthalan (**1a**) with EDA catalyzed by the free cofactor Ir(Me)-MPIX in NaPi buffer (100 mM, pH = 6.0). Without the protein, the free cofactor reaction gave a mixture of products resulting from a single and double insertion of the carbene into the (sp³) C-H bonds of **1a**. Products from a single insertion into the C-H bonds located meta and para to the bromine atom were observed in a 1:1 ratio (Figure 1). Selective carbene insertion into (sp³) C-H bonds catalyzed by transition metal catalysts typically utilize a donor-acceptor carbene precursor.³⁷ There are only a few small molecule catalysts that can handle acceptor-only carbene precursors and they require the substrate as solvent or in excess (**Scheme 1a**).^{38, 39} We reasoned that the pre-organization of substrates within the active site of a protein would afford the opportunity to catalyze the site-selective intermolecular C-H functionalization's using the substrates as the limiting reagents.

To test the capability of engineered Ir(Me)-hemoproteins to differentiate nearly identical C-H bonds and to yield products from insertion of a carbene into just one C-H bond, we conducted reactions of 4-bromophthalan **1a** and EDA with previously-engineered myoglobin (Myo) mutants.^{30, 32} Evaluation of site-selectivity from over one-hundred Ir(Me)-Myo mutants, containing mutations over six active-site residues, from clarified lysate and purified protein reaction screening did not uncover any notable site-selectivity beyond that of the free cofactor. Not dissuaded by the lack of site-selectivity observed with the simple solvent-exposed Myo scaffold, we investigated whether selectivity could be obtained from a natural catalyst's scaffold possessing the more enclosed active site offered by P450 enzymes. Turning to a P450 from the thermophile *Sulfolobus solfataricus*, CYP119, we initially screened selectivity with T213G, C317G, a double mutant that reduced both the size and

hydrogen-bonding ability of the amino acid residue at position 213 and eliminated the native axial heme-ligating cysteine at position 317. In contrast to reactions of the free cofactor, the reactions catalyzed by the double mutant CYP119 enzyme gave only the single carbene insertion products (Figure 1). Although the site-selectivity of the initial experiment was negligible, the improved chemo-selectivity for a single insertion product is clearly imparted by the protein environment.

Scheme 2. Site-selective Intermolecular C-H Insertion Catalyzed by Ir(Me)-MPIX.



Conditions: 5 mM substrate **1a**, 0.2 mol% Ir(Me)-MPIX w/o **CYP119**(C317G), 1 mL solvent (100 mM NaPi, 100 mM NaCl, pH = 6.0 containing 1% vol DMF), slow addition of 10 μ l of EDA (60% Vol in DMF) over 1 hour, 25 °C. Yield refers to the formation of all the carbene insertion products.

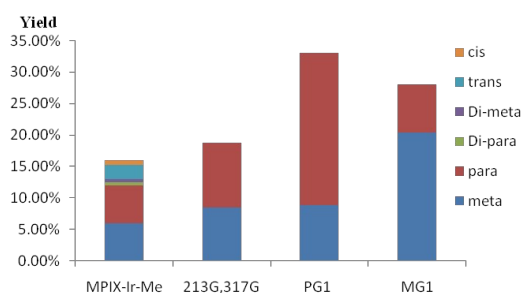


Figure 1. Site-selective Intermolecular C-H Insertion Catalyzed by Ir(Me)-MPIX, and Ir(Me)-CYP119s. **conditions:** 5 mM substrate **1a**, 0.2 mol% Ir(Me)-MPIX, or Ir(Me)-CYP119s (C317G), 1 mL solvent (100 mM NaPi, 100 mM NaCl, pH = 6.0 containing 1% vol DMF), slow addition of 10 μ l of EDA (60% Vol in DMF) over 1 hour, 25 °C. Yield refers to the formation of all the carbene insertion products.

Directed evolution of Ir(Me)-CYP119. To create catalysts for site-selective C-H functionalization of phthalan derivatives, we studied the reaction of 4-bromophthalan **1a** with EDA catalyzed by a mutant library of CYP119 proteins.³⁵ This library was constructed from the double mutant scaffold containing T213G and C317G mutations followed by the introduction of additional mutations at amino acids proximal to the active site (Figure 2). The mutant library was evaluated by screening in 96-well plates. Reactions were conducted by generating a cell lysate solution of Ir(Me)-MPIX-CYP119 by reconstituting each apo-protein mutant variant with a stock solution of Ir(Me)-MPIX cofactor, followed by the addition of phthalan and carbene precursor catalyst stock solutions to the 96-well plates. The ratios of isomeric products from the insertion process were measured by high-throughput GC sampling from 96-well plates.

Results from reaction screening indicated that some members of the CYP119 mutant library catalyzed reactions at one of the two methylene positions of the substituted phthalan with measurable selectivity. For example, the mutant of CYP119 **P_{G1}**(para generation 1: C317G, T213G, V254L, A152F) preferentially formed the product from insertion into the C-H bond para to the bromine atom over that from insertion meta to the bromine atom with a 2.8:1 ratio and 720 TON. In contrast, the mutant of CYP119 **M_{G1}**(meta generation 1: C317G, T213G, V254A, A152L) preferentially formed the product from insertion into the C-H bond meta to the bromine atom with a 1:2.7 ratio and 690 TON (Scheme 2 bar 3 and 4).

Reactions conducted at catalyst loadings lower than 0.05 mol % led to lower conversion, but they also led to lower site-selectivity and gave a mixture of products resulting from a single and double insertion of the carbene into the (sp³) C-H bonds of **1a**. We assumed the lower chemo- and site-selectivities resulted from an instability of the Ir(Me)-CYP119 construct to the reaction conditions, leading to the generation of free cofactor. This decomposition presumably also influenced reactions at higher catalyst loadings. Thus, to improve the stability of the protein to the reaction conditions, we created a library of mutants based on **P_{G1}** and **M_{G1}** by error-prone PCR. These enzymes would contain mutations distal to the active site that could stabilize the Ir(Me)-P450 construct.

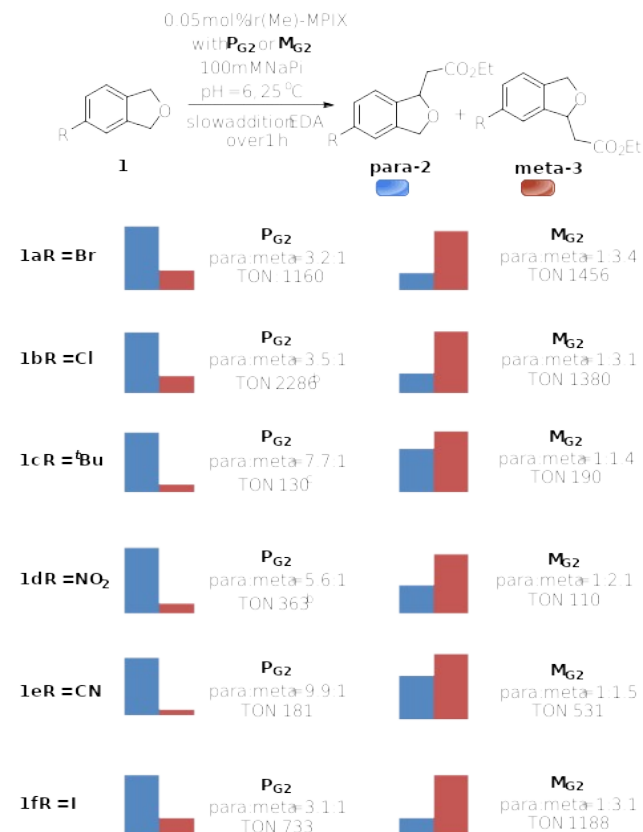
Indeed, three rounds of error-prone PCR led to enzymes that catalyzed the carbene transfer with

greater site-selectivity, in many cases reaching differences in selectivity greater than 10:1 between pairs of mutants reacting at the two methylene positions of **1a** and with higher turnovers. Eleven mutants were identified that catalyzed the reaction to form predominantly the para isomer, and 10 mutants were identified that preferentially formed the meta isomer. These 21 mutants that reacted with selectivities higher than those of the parent mutants were tested for activity across a series of 4-substituted phthalan derivatives, with which small transition metal complexes,³⁶ including the free Ir(Me)-MPIX cofactor, reacted to give insertion products with low selectivity (Figure S54).

Site-Selective Ir(Me)-CYP119 catalyzed carbene insertions into phthalan derivatives. Two of the 21 mutant variants catalyzed the insertion reaction of EDA into a series of 4-substituted phthalan derivatives, having various functional groups, with good TONs and measurable chemo- and site-selectivity for either para or meta isomers. The reaction of 4-bromophthalan **1a** catalyzed by the mutant **P_{G2}**(**P_{G1}**+D177N, H340L) gave a 3.2:1 ratio of constitutional isomers in favor of the para isomer with 1160 TON, while the same reaction catalyzed by the mutant **M_{G2}**(**M_{G1}**+A247S) gave a 1:3.4 ratio of isomers in favor of the meta isomer with 1456 TON (Scheme 3). Similar results were observed for reactions of 4-chlorophthalan **1b**. The reaction catalyzed by the mutant **P_{G2}** formed the product from insertion into the C-H bond para to the chloro-substituent as the major product with a 3.5:1 ratio of constitutional isomers and 2286 TON, while the reaction catalyzed by the mutant **M_{G2}** yielded the meta insertion product in a 1:3.1 ratio and with 1380 TON.

Phthalan **1c** contains a 4-^tBu substituent, which is larger than the halides of **1a** and **1b**. However, the site-selectivity results of reactions of this sterically distinct substrate with CYP119 mutants are similar to those of reactions of **1a** and **1b**. The product from insertion into the C-H bond para to the ^tBu-substituent was favored by the mutant **P_{G2}** in a ratio of 7.7:1 and accomplished with 130 TON. Although the selectivity for the meta C-H bond of **1c** from reactions catalyzed by the mutant **M_{G2}** was lower than that for reaction of **1a** and **1b**, the selectivity, nevertheless, was reversed to 1:1.4 in favor of the meta isomer with up to 190 TON. Thus, we were able, again, to identify a pair of mutants (**P_{G2}** and **M_{G2}**) that react with nearly a >10:1 reversal of site selectivity between mutants for the two types of C-H bonds present in **1c**.

Scheme 3. Scope of Substrates that CYP119 Mutants Reverse Site-selectivity ^a



Conditions a: 5 mM substrate, 0.05 mol% Ir(Me)-MPIX **P_{G2}** or **M_{G2}**, 1 mL solvent (100 mM NaPi, 100 mM NaCl, pH = 6.0 containing 1% vol DMF), slow addition of 10 μ L of EDA (60% vol in DMF) over 1 hour, 25°C. TON refers to the formation of both meta and para C-H bond insertion products. **Conditions b:** 0.02 mol% Ir(Me)-MPIX CYP119 was used.

Nitro-substituted reactants rarely undergo C-H bond functionalization, and the 4-nitro-substituted phthalan **1e** previously did not react with EDA when catalyzed by a small-molecule iridium species.³⁶ However, the reaction of this substrate with EDA catalyzed by an iridium-containing ArM formed the product from insertion into the C-H bond. Although the turnover numbers from this reaction were lower than those from reactions of the other substrates, the reaction of 4-nitrophthalan **1d** catalyzed by the mutant **P_{G2}** formed the product from reaction of the C-H bond para to the nitro-substituent as the major isomer with a 5.6:1 ratio over that from reaction at the meta C-H bond (363 TON). The same reaction catalyzed by the mutant **M_{G2}** preferentially formed the product from reaction at the meta C-H bond with a 1:2.1 ratio of constitutional isomers. A similar preference for

reaction with the C-H bond para to the substituent was observed from the reaction of 4-cyanophthalan **1e** catalyzed by **P_{G2}** with a 9.9:1 ratio over the reaction at the methylene C-H bond meta to the cyano-substituent (181 TON). The selectivity was reversed to 1:1.5 in favor of reaction at the C-H bond meta to the cyano-substituent for the reaction catalyzed by the mutant **M_{G2}** (531) TON, again demonstrating a 10:1 reversal of site-selectivity for a pair of mutant ArMs. Reaction of the 4-iodophthalan **1f** also occurred with good selectivity for insertion at the para position (3.1:1, 733 TON) when catalyzed by the mutant **P_{G2}**, whereas the same selectivity in favor of reaction at the meta position (1:3.1) was observed for the reaction catalyzed by mutant **M_{G2}** (1188 TON).

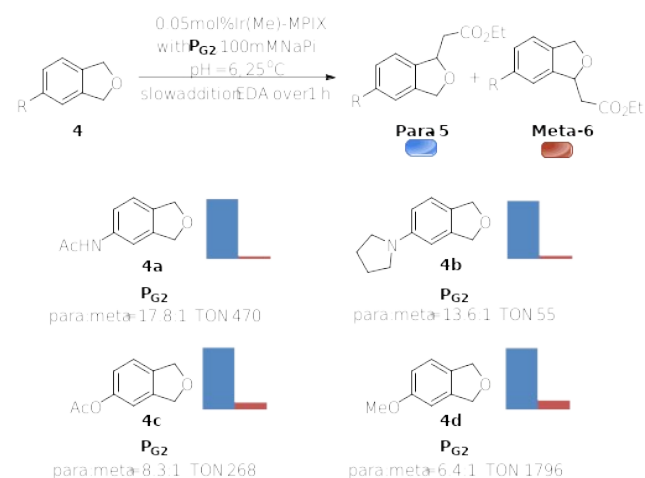
Substrates for which the mutant enzymes selectively react at the para C-H bond. In addition to reacting site-selectively for one C-H bond over another similar C-H bond, the ArMs we report react with high chemoselectivity toward carbene insertion into C-H bonds over N-H bonds. The reaction of amide-substituted phthalan **4a** catalyzed by the free Ir(Me)-MPIX cofactor in NaPi buffer occurred with low conversion (<5%) and gave <2% amount of product from C-H insertion. The same reaction catalyzed by **P_{G2}** formed the product from insertion of the carbene into the C-H bond with 17.8:1 selectivity for the C-H bond para to the amide-substituent and 470 TON. No product from insertion of the carbene into the N-H bond was observed from reactions catalyzed by any of the Ir(Me)-CYP119 constructs.

Likewise, the products of the reactions of EDA with amine substituted phthalan **4b** catalyzed by the free Ir(Me)-porphyrin and artificial enzyme were distinct. The reaction of EDA with **4b** catalyzed by Ir(Me)-MPIX gave the product from addition to the nitrogen to form a quaternary ammonium salt. However, the reaction catalyzed by the mutant **P_{G2}** occurred with high chemo- and site-selectivity for the C-H bond para to the amine-substituent (13.6:1 para selectivity and 55 TON).

The reactions of EDA with ester substituted phthalan **4c** and ether substituted phthalan **4d** catalyzed by **P_{G2}** also occurred with higher site-selectivity compared to the reactions catalyzed by free-cofactor. The reaction catalyzed by Ir(Me)-MPIX occurred with low TONs and almost no selectivity for the methylene units para and meta to the ester- and ether-substituents (Figure S55). However, selectivity of 8.3:1 was observed for reaction at the methylene position para to the

ester-substituent of **4c** when catalyzed by the mutant **P_{G2}** (268 TON). The reaction of **4d** occurred with 1796 TON and 6.4:1 selectivity for the methylene para to the methoxy-substituent. Reactions of **4c** and **4d** illustrate the potential of ArMs to control site-selectivity compared to the reaction with free cofactor.

Scheme 4. Scope of Substrates for Which Mutant Enzymes are Selective for Reaction at the Para C-H Bond.^a

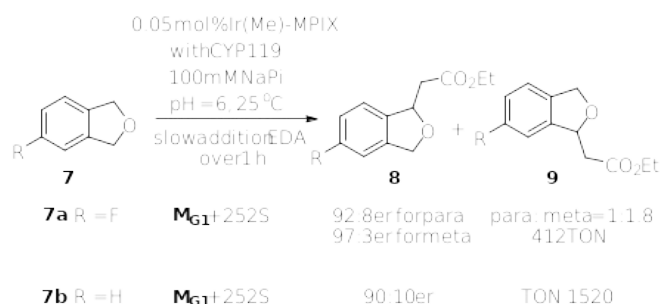


Conditions a: 5 mM substrate, 0.05mol% Ir(Me)-**P_{G2}**, 1 mL solvent (100 mM NaPi, 100 mM NaCl, pH = 6.0 containing 1% vol DMF), slow addition of 10 μ l of EDA (60% Vol in DMF) over 1 hour, 25°C. TON refers to the formation of both meta and para C-H bond insertion products.

Enantioselective intermolecular C-H insertion catalyzed by Ir(Me)-CYP119. Our results show that the environment within the ArM can control the number of insertion events, site-selectivity of the atom transfer position, and tolerate several competitive functional groups. We have shown previously that C-H insertion reactions catalyzed by Ir(Me)-P450s can occur in an enantioselective fashion.³⁵ However, because the current work is focused on assessing the ability of ArMs to catalyze reactions with site-selectivity, we did not perform extensive evolution to enhance enantioselectivity. Nevertheless, some of the reactions did occur with substantial enantioselectivity when catalyzed by members of the mutant library. For example, the reactions of EDA with 4-fluorophthalan (**7a**) occurred to give the major constitutional isomer in 97:3 er and the minor isomer in 92:8 er when catalyzed by **M_{G1}**+P252S (Scheme 5). The ratio of constitutional isomers was 1:2.0 in favor of the meta isomer. Although this site-selectivity is lower than that of reactions of other substituted phthalans, it does show that

the enzyme can react with significant site-selectivity and enantioselectively for two C-H bonds that differ only by a remote fluorine versus hydrogen on the aryl ring. A fluorine atom has the closest size to hydrogen of any atom, and has the closest Hammett parameter to hydrogen of nearly any substituent.⁴⁰ In comparison with our prior results from the reaction of phthalan and EDA catalyzed by an Ir(Me)-P450 (84:16 er, 324 TON),³⁵ an increase in enantioselectivity and reactivity was observed from the same reaction catalyzed by the mutant **M_{G1}**+252S with 90:10 er and 1520 TON.¹⁶

Scheme 5. Enantioselective Intermolecular C-H Insertion Catalyzed by Ir(Me)-CYP119.



Conditions: 5 mM substrate, 0.05 mol% Ir(Me)-CYP119 with a series of mutants, 1 mL solvent (100 mM NaPi, 100 mM NaCl, pH = 6.0 containing 1% vol DMF), slow addition of 10 μ L of EDA (60% Vol in DMF) over 1 hour, 25 °C. TON refers to the formation of both meta and para C-H bond insertion products.

Stability of a series of Ir(Me)-P450 mutants.

Through structural analysis it was revealed that the majority of mutations (A152, T213, V254 and C317) are located within the active site; however, some mutations, especially those generated by error-prone PCR (D177, H340 and A247), are located far from the active site in both **P_{G2}** and **M_{G2}** (Figure 2). As noted previously in this paper, these additional mutations appear to enhance the stability of the Ir(Me)-P450 construct because they have a larger influence on turnover number than on site-selectivity (Scheme 3 and 4). To assess this line of reasoning, we evaluated the stability of the Ir(Me)-CYP119 construct to temperature and reaction components.

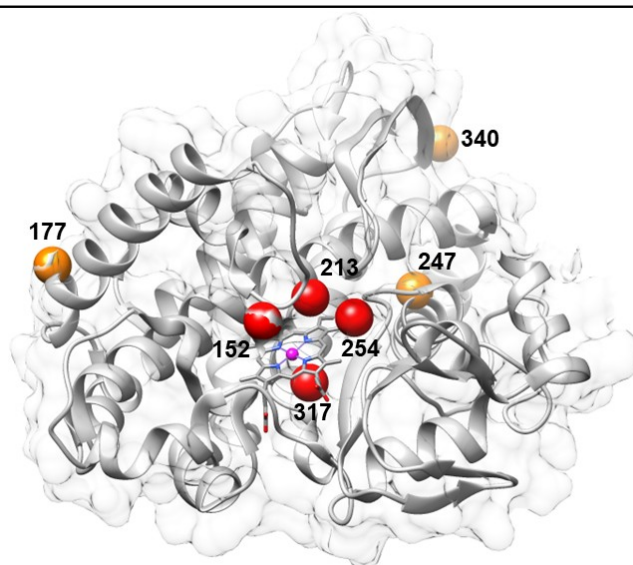


Figure 2. Structure of WT Fe-CYP119 (image prepared in Chimera from PDB 1IO7).⁴¹ Red label: mutant positions that were generated by site-directed mutagenesis (A152, T213, V254, C317); Orange label: additional positions generated by error-prone PCR for para selectivity (D177 and H340) and meta selectivity (A247).

To assist our evaluation of the effect of the mutations distal to the active site, we generated two mutants, **M_{Max}** (**M_{G2}** + D177N, H340L) and **P_{max}** (**P_{G2}** + 247S), which contain all of the mutations peripheral to the active site in either **M_{G2}** or **P_{G2}**. Like **P_{G1}**, **P_{max}** catalyzed the reaction of **1a** with a similar site selectivity ratio of 3:1 in favor of the para isomer, but the turnover number of the reaction catalyzed by **P_{max}** (1450 TON) was twice as high as that of the reaction catalyzed by **P_{G1}** (720 TON) (Figure 3). Likewise, the site selectivity of the reaction catalyzed by **M_{max}** was similar to that of the reaction catalyzed by **M_{G1}** (1:2.7 for **M_{G1}** vs. 1:3.5 for CYP **M_{max}**), but the TON of the reaction catalyzed by **M_{max}** (1834 TON) was more than three times higher than that of the parent **M_{G1}** mutant (580 TON) (Figure 3). These results further support the conclusion that the mutations resulting from error-prone PCR (177N, 340L and 247S) stabilize the Ir(Me)-P450 construct during the course of the reaction.

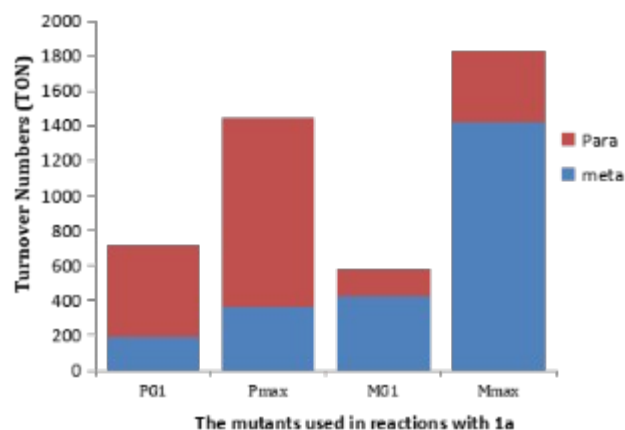


Figure 3. Activity studies of different mutants with slow addition of EDA with **1a**. Conditions: 5 mM substrate, 0.05 mol% Ir(Me)-PIX CYP119 with **P_{G1}**, **P_{max}**, **M_{G1}**, and **M_{max}**, 1 mL solvent (100 mM NaPi, 100 mM NaCl, pH = 6.0 containing 1% vol DMF), slow addition of 10 μ L of EDA (60% Vol in DMF) over 1 hour, 25 $^{\circ}$ C. TON refers to the formation of both meta and para C-H bond insertion products

Recently it was reported that a major deactivation pathway during cyclopropanation of alkenes catalyzed by repurposed heme enzymes and ruthenium-containing ArMs involves carbene transfer into the amino acid residues of the host protein.^{31, 42} Because Ir(Me)-MPIX is an extremely active catalyst for carbene transfers, we suspected that this degradation pathway could limit the TON of the Ir(Me)-P450 construct and that these distal mutations might be retarding this degradation. However, the reaction of EDA with all of the mutants of Ir(Me)-PIX CYP119 or reactions of EDA with 4-bromophthalan **1a** catalyzed by all the mutants of Ir(Me)-PIX CYP119 did not reveal masses corresponding to addition of EDA to the protein or addition of the methyl group of the Ir(Me) cofactor to the protein (Figure S73-S78). Instead, the carbene undergoes rapid formation of fumarate and maleate (Figure S79). Perhaps the high rate of dimerization prevents reaction of the carbene unit with amino acid residues of the protein scaffold.

Prior studies on repurposed and artificial metalloenzymes for carbene transfer showed that the CD spectrum changed upon exposure to EDA.⁴² Thus, we measured the CD spectra of **P_{G1}**, **P_{G2}**, **P_{max}**, **M_{G1}**, **M_{G2}** and **M_{max}** in the apo- and in the holo-Ir(Me)-CYP119 forms before and after exposure to EDA. All of these CD spectra are indistinguishable from each other. No notable differences between the CD spectra of the apo- and holo-CYP119 form were observed, or from

that of the WT-CYP119 protein.^{43, 44} The α -helicity ($[\theta]_{222}/[\theta]_{208}$) ratio was within 1.00 and 0.96 for all of the Ir(Me)-P450 constructs and similar to what was reported for the WT-CYP119 protein⁴³ (Figures S61-S72).⁴⁵

The melting temperatures (T_m) of a series of mutants were measured by differential scanning calorimetry, and no significant differences among the T_m values of the mutants were observed. The average T_m value was 67.5 $^{\circ}$ C with a standard deviation of 0.2 $^{\circ}$ C among all the mutants (Table S1 and Figures S84-S89). The reported T_m value of WT-CYP119 is 91.9 $^{\circ}$ C. It is not surprising that the T_m values of the mutants of Ir(Me)-MPIX CYP119 are lower than that of WT-CYP119, given the mutations and exchange of an axially-ligated Fe-heme cofactor for a non-ligated artificial Ir(Me)-MPIX cofactor.⁴³ The similarity of these T_m values indicate that mutations in the active site or in the periphery of the protein have little effect on the folding and thermal stability of the CYP119 construct. Therefore, the higher TON of the systems containing peripheral mutations seems to result from greater stability of the Ir(Me)-P450 constructs during catalysis.

To probe the stability of CYP119s toward the reaction components (substrate, product, solvent, etc.), Ir(Me)-CYP119 mutants were treated with components of the reaction mixture for one hour prior to initiating reactions (Figures S57-S59). This pretreatment of Ir(Me)-CYP119s with the reaction products influenced the selectivity of the reaction. For example, reactions of 4-bromophthalan **1a** with EDA conducted after treating Ir(Me)-**P_{G1}** or Ir(Me)-**M_{G1}** with 4-chlorophthalan-product **2b** for one hour occurred with negligible site-selectivity, whereas the same reaction conducted after treating Ir(Me)-**M_{max}** or Ir(Me)-**P_{max}** with **2b** for one hour occurred with site-selectivities of 1:2 favoring the meta isomer and 1.8:1 favoring the para isomer, respectively (Figures S60). These results imply that the peripheral mutations stabilize the enzyme toward the C-H insertion products. This trend in effect of product on selectivity is similar to the trend in stability of the protein determined independently by CD (Figures S80-S83). Although difficult to correlate quantitatively, we found that the concentrations of the proteins containing peripheral mutations (**P_{max}** and **M_{max}**) were higher than those of the parent mutants **P_{G1}** and **M_{G1}** after exposure of equal concentrations of the Ir(Me)-P450 proteins to acetate-substituted product **6c**.⁴⁶

CONCLUSION

In summary, artificial metalloenzymes containing a noble metal porphyrin (Ir(Me)-CYP119) are capable of catalyzing the intermolecular insertion of acceptor-only carbenes into nearly equivalent C–H bonds with up to 17.8:1 site-selectivity for a series of 4-substituted phthalans. Through laboratory evolution a nearly 10:1 ratio between either the meta or para constitutional isomers can be obtained using two distinct Ir(Me)-CYP119 mutants. Although not the primary focus of this study, enantioselectivity of 98:2 can be obtained with the current mutant library for the insertion of EDA into 4-fluorophthalan. The insertion reactions catalyzed by Ir(Me)-P450 tolerated a series of functional groups that proved difficult or impossible to accommodate with small molecule catalysts. Furthermore, unlike other ArMs or repurposed heme enzymes, the Ir(Me)-P450s are less susceptible to deactivation from EDA insertion into the protein framework. This work highlights important types of selectivities that are difficult to achieve with small-molecule catalysts but can be readily achieved by combining a highly-active transition metal catalyst with a readily-evolvable protein framework.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>. Experimental details and procedures, spectra for all unknown compounds (PDF)

AUTHOR INFORMATION

Corresponding Author

*jhartwig@berkeley.edu

ORCID

John F. Hartwig: 0000-0002-4157-468X

Notes

The authors declare no competing financial interests

ACKNOWLEDGMENT

This work was supported by the Director, Office of Science, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Y. G. thanks a joint postdoc fellowship from Pharmaron and Shanghai Institute of Organic Chemistry (SIOC). S.N.N thanks the NIH (F32-GM126652) and the Burroughs Wellcome fund (PDEB). Z. L thanks to National Science Scholarship from the Singapore Agency for Science, Technology and Research. We thank the molecular Foundry for their assistance in DSC measurements. Work at the Molecular Foundry was supported by the Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

REFERENCES

1. Yamaguchi, J.; Yamaguchi, A. D.; Itami, K., C–H Bond Functionalization: Emerging Synthetic Tools for Natural Products and Pharmaceuticals. *Angew. Chem. Int. Ed.* **2012**, *51*, 8960-9009.
2. Brückl, T.; Baxter, R. D.; Ishihara, Y.; Baran, P. S., Innate and Guided C–H Functionalization Logic. *Acc. Chem. Res.* **2012**, *45*, 826-839.
3. Hartwig, J. F., Evolution of C–H Bond Functionalization from Methane to Methodology. *J. Am. Chem. Soc.* **2016**, *138*, 2-24.
4. Das, S.; Incarvito, C. D.; Crabtree, R. H.; Brudvig, G. W., Molecular Recognition in the Selective Oxygenation of Saturated C–H Bonds by a Dimanganese Catalyst. *Science* **2006**, *312*, 1941.
5. Engle, K. M.; Mei, T.-S.; Wasa, M.; Yu, J.-Q., Weak Coordination as a Powerful Means for Developing Broadly Useful C–H Functionalization Reactions. *Acc. Chem. Res.* **2012**, *45*, 788-802.
6. Liao, K.; Pickel, T. C.; Boyarskikh, V.; Bacsá, J.; Musaev, D. G.; Davies, H. M. L., Site-selective and stereoselective functionalization of non-activated tertiary C–H bonds. *Nature* **2017**, *551*, 609-613.
7. Anding, B. J.; Dairo, T. O.; Woo, L. K., Reactivity Comparison of Primary Aromatic Amines and Thiols in E–H Insertion Reactions with Diazoacetates Catalyzed by Iridium(III) Tetratolylporphyrin. *Organometallics* **2017**, *36*, 1842-1847.
8. Dairo, T. O.; Woo, L. K., Scope and Mechanism of Iridium Porphyrin-Catalyzed S–H Insertion Reactions between Thiols and Diazo Esters. *Organometallics* **2017**, *36*, 927-934.
9. Che, C.-M.; Lo, V. K.-Y.; Zhou, C.-Y.; Huang, J.-S., Selective functionalisation of saturated C–H bonds with metalloporphyrin catalysts. *Chem. Soc. Rev.* **2011**, *40*, 1950-1975.
10. Narayan, A. R. H.; Jiménez-Osés, G.; Liu, P.; Negretti, S.; Zhao, W.; Gilbert, M. M.; Ramabhadran, R. O.; Yang, Y.-F.; Furan, L. R.; Li, Z.; Podust, L. M.; Montgomery, J.; Houk, K. N.; Sherman, D. H., Enzymatic hydroxylation of an unactivated methylene C–H bond guided by molecular dynamics simulations. *Nat. Chem.* **2015**, *7*, 653-660.
11. Negretti, S.; Narayan, A. R. H.; Chiou, K. C.; Kells, P. M.; Stachowski, J. L.; Hansen, D. A.; Podust, L. M.; Montgomery, J.; Sherman, D. H., Directing Group-Controlled Regioselectivity in an Enzymatic C–H Bond Oxygenation. *J. Am. Chem. Soc.* **2014**, *136*, 4901-4904.
12. Isin, E. M.; Guengerich, F. P., Complex reactions catalyzed by cytochrome P450

- enzymes. *Biochim. Biophys. Acta* **2007**, *1770*, 314-329.
13. Arnold, F. H., Directed Evolution: Bringing New Chemistry to Life. *Angew. Chem. Int. Ed.* **2018**, *57*, 4143-4148.
14. Packer, M. S.; Liu, D. R., Methods for the directed evolution of proteins. *Nature Reviews Genetics* **2015**, *16*, 379.
15. Sun, Z.; Wikmark, Y.; Bäckvall, J.-E.; Reetz, M. T., New Concepts for Increasing the Efficiency in Directed Evolution of Stereoselective Enzymes. *Chem. Eur. J.* **2016**, *22*, 5046-5054.
16. Zhang, R. K.; Chen, K.; Huang, X.; Wohlschlager, L.; Renata, H.; Arnold, F. H., Enzymatic assembly of carbon-carbon bonds via iron-catalysed sp³ C-H functionalization. *Nature* **2019**, *565*, 67-72.
17. Hammer, S. C.; Knight, A. M.; Arnold, F. H., Design and evolution of enzymes for non-natural chemistry. *Curr. Opin. Green Sust. Chem.* **2017**, *7*, 23-30.
18. Brandenberg, O. F.; Fasan, R.; Arnold, F. H., Exploiting and engineering hemoproteins for abiological carbene and nitrene transfer reactions. *Curr. Opin. Biotechnol.* **2017**, *47*, 102-111.
19. Schwizer, F.; Okamoto, Y.; Heinisch, T.; Gu, Y.; Pellizzoni, M. M.; Lebrun, V.; Reuter, R.; Köhler, V.; Lewis, J. C.; Ward, T. R., Artificial Metalloenzymes: Reaction Scope and Optimization Strategies. *Chem. Rev.* **2018**, *118*, 142-231.
20. Ohashi, M.; Koshiyama, T.; Ueno, T.; Yanase, M.; Fujii, H.; Watanabe, Y., Preparation of Artificial Metalloenzymes by Insertion of Chromium(III) Schiff Base Complexes into Apomyoglobin Mutants. *Angew. Chem. Int. Ed.* **2003**, *42*, 1005-1008.
21. Carey, J. R.; Ma, S. K.; Pfister, T. D.; Garner, D. K.; Kim, H. K.; Abramite, J. A.; Wang, Z.; Guo, Z.; Lu, Y., A Site-Selective Dual Anchoring Strategy for Artificial Metalloprotein Design. *J. Am. Chem. Soc.* **2004**, *126*, 10812-10813.
22. Oohora, K.; Meichin, H.; Zhao, L.; Wolf, M. W.; Nakayama, A.; Hasegawa, J.-y.; Lehnert, N.; Hayashi, T., Catalytic Cyclopropanation by Myoglobin Reconstituted with Iron Porphycene: Acceleration of Catalysis due to Rapid Formation of the Carbene Species. *J. Am. Chem. Soc.* **2017**, *139*, 17265-17268.
23. Heinisch, T.; Ward, T. R., Artificial Metalloenzymes Based on the Biotin-Streptavidin Technology: Challenges and Opportunities. *Acc. Chem. Res.* **2016**, *49*, 1711-1721.
24. Liang, A. D.; Serrano-Plana, J.; Peterson, R. L.; Ward, T. R., Artificial Metalloenzymes Based on the Biotin-Streptavidin Technology: Enzymatic Cascades and Directed Evolution. *Acc. Chem. Res.* **2019**, *52*, 585-595.
25. Reetz, M. T., Directed Evolution of Artificial Metalloenzymes: A Universal Means to Tune the Selectivity of Transition Metal Catalysts? *Acc. Chem. Res.* **2019**, *52*, 336-344.
26. Lewis, J. C., Metallopeptide catalysts and artificial metalloenzymes containing unnatural amino acids. *Curr. Opin. Chem. Biol.* **2015**, *25*, 27-35.
27. Lewis, J. C., Beyond the Second Coordination Sphere: Engineering Dirhodium Artificial Metalloenzymes To Enable Protein Control of Transition Metal Catalysis. *Acc. Chem. Res.* **2019**, *52*, 576-584.
28. Mirts, E. N.; Bhagi-Damodaran, A.; Lu, Y., Understanding and Modulating Metalloenzymes with Unnatural Amino Acids, Non-Native Metal Ions, and Non-Native Metallocofactors. *Acc. Chem. Res.* **2019**, *52*, 935-944.
29. Natoli, S. N.; Hartwig, J. F., Noble-Metal Substitution in Hemoproteins: An Emerging Strategy for Abiological Catalysis. *Acc. Chem. Res.* **2019**, *52*, 326-335.
30. Key, H. M.; Dydio, P.; Clark, D. S.; Hartwig, J. F., Abiological catalysis by artificial haem proteins containing noble metals in place of iron. *Nature* **2016**, *534*, 534-537.
31. Wolf, M. W.; Vargas, D. A.; Lehnert, N., Engineering of RuMb: Toward a Green Catalyst for Carbene Insertion Reactions. *Inorg. Chem.* **2017**, *56*, 5623-5635.
32. Sreenilayam, G.; Moore, E. J.; Steck, V.; Fasan, R., Metal Substitution Modulates the Reactivity and Extends the Reaction Scope of Myoglobin Carbene Transfer Catalysts. *Adv. Synth. Catal.* **2017**, *359*, 2076-2089.
33. Key, H. M.; Dydio, P.; Liu, Z.; Rha, J. Y. E.; Nazarenko, A.; Seyedkazemi, V.; Clark, D. S.; Hartwig, J. F., Beyond Iron: Iridium-Containing P450 Enzymes for Selective Cyclopropanations of Structurally Diverse Alkenes. *ACS Cent. Sci.* **2017**, *3*, 302-308.
34. Dydio, P.; Key, H. M.; Hayashi, H.; Clark, D. S.; Hartwig, J. F., Chemoselective, Enzymatic C-H Bond Amination Catalyzed by a Cytochrome P450 Containing an Ir(Me)-PIX Cofactor. *J. Am. Chem. Soc.* **2017**, *139*, 1750-1753.
35. Dydio, P.; Key, H. M.; Nazarenko, A.; Rha, J. Y. E.; Seyedkazemi, V.; Clark, D. S.; Hartwig, J. F., An artificial metalloenzyme with the kinetics of native enzymes. *Science* **2016**, *354*, 102-106.
36. Weldy, N. M.; Schafer, A. G.; Owens, C. P.; Herting, C. J.; Varela-Alvarez, A.; Chen, S.; Niemeyer, Z.; Musaev, D. G.; Sigman, M. S.; Davies, H. M. L.; Blakey, S. B., Iridium(III)-bis(imidazolyl)phenyl catalysts for enantioselective C-H functionalization with ethyl diazoacetate. *Chem. Sci.* **2016**, *7*, 3142-3146.
37. Doyle, M. P.; Duffy, R.; Ratnikov, M.; Zhou, L., Catalytic Carbene Insertion into C-H Bonds. *Chem. Rev.* **2010**, *110*, 704-724.

-
38. Díaz-Requejo, M. M.; Belderrain, T. R.; Nicasio, M. C.; Trofimenko, S.; Pérez, P. J., Intermolecular Copper-Catalyzed Carbon–Hydrogen Bond Activation via Carbene Insertion. *J. Am. Chem. Soc.* **2002**, *124*, 896-897.
39. Weldy, N. M.; Schafer, A. G.; Owens, C. P.; Herting, C. J.; Varela-Alvarez, A.; Chen, S.; Niemeyer, Z.; Musaev, D. G.; Sigman, M. S.; Davies, H. M. L.; Blakey, S. B., Iridium(iii)-bis(imidazolyl)phenyl catalysts for enantioselective C–H functionalization with ethyl diazoacetate. *Chem Sci* **2016**, *7*, 3142-3146.
40. Hansch, C.; Leo, A.; Taft, R. W., A survey of Hammett substituent constants and resonance and field parameters. *Chem. Rev.* **1991**, *91*, 165-195.
41. Park, S.-Y.; Yamane, K.; Adachi, S.-i.; Shiro, Y.; Weiss, K. E.; Sligar, S. G., Crystallization and preliminary X-ray diffraction analysis of a cytochrome P450 (CYP119) from *Sulfolobus solfataricus*. *Acta Crystallogr., Sect D: Biol. Crystallogr.* **2000**, *56*, 1173-1175.
42. Renata, H.; Lewis, R. D.; Sweredoski, M. J.; Moradian, A.; Hess, S.; Wang, Z. J.; Arnold, F. H., Identification of Mechanism-Based Inactivation in P450-Catalyzed Cyclopropanation Facilitates Engineering of Improved Enzymes. *J. Am. Chem. Soc.* **2016**, *138*, 12527-12533.
43. Park, S.-Y.; Yamane, K.; Adachi, S.-i.; Shiro, Y.; Weiss, K. E.; Maves, S. A.; Sligar, S. G., Thermophilic cytochrome P450 (CYP119) from *Sulfolobus solfataricus*: high resolution structure and functional properties. *J. Biol. Inorg. Chem.* **2002**, *91*, 491-501.
44. We cannot rule out transfer of the carbene to the porphyrin unit. We have been unable to isolate the metal-porphyrin cofactor after the reaction for analysis by mass spectroscopy.
45. Banerjee, R.; Sheet, T., Ratio of ellipticities between 192 and 208 nm (R1): An effective electronic circular dichroism parameter for characterization of the helical components of proteins and peptides. *Proteins* **2017**, *85*, 1975-1982.
46. **6c** was chosen because it has the weakest absorption at 280nm and high solubility compared to other 4-pthalan products.

